Fasting enhances glycogen synthase activation in hepatocytes from insulin-resistant genetically obese (*fa/fa*) rats

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Glycogen synthase activation and phosphorylase inactivation by glucose were studied in hepatocytes isolated from fed or overnight-fasted lean or genetically obese (*fa/fa*) rats. In cells from fed animals, both the time course and dose-response to glucose of synthase activation were the same in both groups, despite higher levels of phosphorylase *a* in hepatocytes from obese animals. In contrast, in cells from fasted obese animals synthase activation with or without glucose was enhanced severalfold over that of lean controls, despite similar levels of phosphorylase *a* and of total (*a* + *b*) synthase activities. In both nutritional conditions glucose 6-phosphate concentrations were 2–3-fold higher in obese-rat hepatocytes than in lean-rat cells. In addition, synthase activation was transient in the fasted lean group, but was sustained in obese-rat hepatocytes. The rate of synthase activation was, however, comparable in lean- and obese-rat liver Sephadex G-25 filtrates, irrespective of the nutritional state of the donor rats. It is concluded that enhanced synthase activation in hepatocytes from starved obese rats might be due to an unbalanced synthase interconversion brought about by elevated glucose 6-phosphate concentrations and impaired kinase [van de Werve & Massillon (1990) *Biochem. J.* 269, 795–799], rather than to an intrinsic change in synthase phosphatase.

INTRODUCTION

Because genetically obese (*fa/fa*) rats are hyperinsulinaemic, even when fasted overnight, and because their portal-blood glucose increases markedly after a meal, one should expect liver glycogen synthesis in that condition to be enhanced as compared with lean controls. This is, however, not the case, since similar levels of glycogen synthase *a* and rates of glycogen accumulation were observed in fasted-refed lean and *fa/fa* rats (van de Werve & Jeanrenaud, 1987). These unexpected findings were tentatively explained by an increased turnover of glycogen and/or a defect in the interconversion of phosphorylase consequent to the reported (Terrettaz *et al.*, 1986) hepatic insulin resistance in *fa/fa* rats *in vivo*. As glycogen synthase and phosphorylase phosphatase activities *in vitro* were found to be increased in liver from obese (*fa/fa*) rats (Margolis, 1987), it was decided to study the interconversion of phosphorylase and synthase in the intact cell. We found that synthase activation was markedly enhanced in hepatocytes from obese rats, but only in the fasted state, and was accompanied by higher levels of glucose 6-phosphate (Glc-6-P).

EXPERIMENTAL

Handling of animals

Fed or overnight-fasted female 10-week-old lean (*FA/fa*?) (about 200 g body wt.) and genetically obese (*fa/fa*) rats (about 250 g body wt.) were purchased from Charles River (Canada). Portal-blood and liver sampling were performed as described previously (van de Werve & Jeanrenaud, 1987).

Preparation and incubation of hepatocytes

Hepatocytes were prepared (van de Werve, 1980) between 09:00 and 11:00 h from the fed or overnight-fasted lean or obese rats defined above, and were incubated at 37 °C in Krebs–Henseleit medium containing various concentrations of glucose as indicated. At different incubation times, samples (100 µl) of cell suspension (50 mg/ml) were frozen in liquid N₂ and stored at –65 °C pending further determinations.

Preparation and incubation of liver filtrates

Fed or overnight-fasted lean and obese rats were decapitated, and the livers removed and homogenized in a Potter–Elvehjem tube in 5 vol. of ice-cold 0.25 M-sucrose/0.5 mM-dithiothreitol/50 mM-imidazole adjusted to pH 7.4 at room temperature. The homogenate was centrifuged for 10 min at 11 000 g, and 500 µl of the supernatant was filtered on a Sephadex G-25 (medium grade) column (15 ml gel bed) equilibrated in the homogenization buffer. The fraction (700 µl, containing 15–20 mg of protein/ml) eluted with haemoglobin (filtrate) was collected and incubated at 20 °C with 70 µl of 50 mM-(NH₄)₂SO₄. At different incubation times, samples (50 µl) of filtrate were frozen in liquid N₂ and stored at –65 °C pending further determinations.

Assays of enzymes and metabolites

Glycogen synthase and phosphorylase activities were assayed in homogenates of thawed cell suspensions and of filtrates as described previously (Hue *et al.*, 1975). Glycogen was measured by the method of Chan & Exton (1976). For the measurement of metabolites, 1 ml of cells plus medium was centrifuged at 16 000 g for 10 s and the supernatant was removed. The cell pellet was immediately resuspended in 1 ml of 10% (w/v) trichloroacetic acid and further processed for the standard spectrophotometric assay of Glc-6-P and UDP-glucose (UDP-Glc) (Bergmeyer, 1974). Such a prior separation of cells from medium for the measurement of hepatocyte metabolites was recommended by Van Schaftingen *et al.* (1987), given the significant proportion of extracellular metabolites found in the medium. Plasma glucose and insulin were measured as described by van de Werve & Jeanrenaud (1987).

Statistical analysis

Statistical analysis was performed by using Student's *t*-test for unpaired samples: *P* < 0.05 was considered statistically significant.

Abbreviations used: Glc-6-P, glucose 6-phosphate; UDP-Glc, UDP-glucose.

RESULTS

Characteristics of lean and obese rats

Table 1 shows the portal-vein glucose and insulin levels as well as the liver glycogen contents of the different groups of rats investigated. It is noteworthy that fasting did not deplete glycogen as much in livers of obese as in lean animals, and that these obese rats display fasting hyperinsulinaemia and normoglycaemia as we observed previously (van de Werve & Jeanrenaud, 1987).

Table 1. Liver glycogen, portal-vein glucose and insulin levels of fed and 15 h-fasted lean and obese rats

Values are means $\pm$ S.E.M. for six animals per group and nutritional condition. All parameters (fasted lean versus obese) are statistically different ($P < 0.01$; unpaired t test), except portal glucose.

	Lean		Obese	
	Fasted	Fed	Fasted	Fed
Glycogen (mg/g of liver)	0.54 $\pm$ 0.34	58.3 $\pm$ 3.2	14 $\pm$ 1.4	71.5 $\pm$ 3.7
Portal glucose (mg/dl)	73 $\pm$ 5	102 $\pm$ 2	82 $\pm$ 6	128 $\pm$ 10
Portal insulin (ng/ml)	4.3 $\pm$ 1.6	2.8 $\pm$ 0.7	37 $\pm$ 2	19 $\pm$ 3

Time course of phosphorylase inactivation and glycogen synthase activation in hepatocytes

Fig. 1 shows the time-dependent inactivation of phosphorylase and activation of synthase in freshly isolated hepatocytes from fed lean and obese rats. Increasing glucose concentrations enhanced the rate and extent of phosphorylase inactivation and that of synthase activation similarly in liver cells from both groups of animals. The amount of phosphorylase in the a form was, however, higher in obese-rat hepatocytes ($\blacktriangle$ versus $\triangle$) in all experimental conditions. This difference was not explained by changes in total ($a+b$) phosphorylase between the two groups (see legend of Fig. 1).

In hepatocytes from fasted rats (zero times, Fig. 2), the phosphorylase a levels were also higher in the obese group, but tended to be inactivated with time towards the same value as that reached in the lean group. Synthase activation was markedly enhanced in the absence as well as in the presence of glucose in fasted obese-rat hepatocytes (Fig. 2), without change in total ($a+b$) activity (see legend of Fig. 2).

In addition, comparison of the lower panels of Figs. 1 and 2 shows that the kinetics of synthase interconversion differed with the nutritional condition. The maximal activation of synthase was reached after 30 min, and was maintained for up to 60 min in cells from fed lean and obese animals. In hepatocytes from fasted lean and obese rats, maximal synthase values were attained much earlier (at 10 min), and the activation was transient in nature, with an almost complete reversal after 60 min of incubation in the lean group and a similar decrease in the absolute value of synthase in the obese group.

Fig. 1. Time course of synthase activation and phosphorylase inactivation in hepatocytes from fed lean and obese rats incubated with various concentrations of glucose

The activities of synthase a ($\circ$, $\bullet$) and of phosphorylase a ($\triangle$, $\blacktriangle$) were measured in hepatocytes from lean ($\circ$, $\triangle$) and obese ($\bullet$, $\blacktriangle$) rats incubated for different times up to 60 min in the absence or presence of various concentrations of glucose as indicated. Values for phosphorylase and synthase are means $\pm$ S.E.M. of three individual preparations of hepatocytes per animal group. All values of phosphorylase, but not of synthase, were statistically different from each other between the lean and obese groups. Total ($a+b$) phosphorylase (15 ± 0.8 and 22.7 ± 1.0 units/g of liver) and synthase (1.5 ± 0.08 and 1.34 ± 0.09 units/g of liver) values for lean and obese respectively were not statistically different.

Fig. 2. Time course of synthase activation and phosphorylase inactivation in hepatocytes from fasted lean and obese rats incubated with various concentrations of glucose

Same legend and symbols as in Fig. 1. All values of synthase and those of phosphorylase at zero time were statistically different from each other between the lean and obese groups, except synthase at zero time and at 10 min of incubation in the absence and presence of 10 mM-glucose. Total ($a+b$) phosphorylase (17.9 ± 2.1 and 23.9 ± 1.3 units/g of liver) and synthase (1.78 ± 0.2 and 1.31 ± 0.1 units/g of liver) values for lean and obese respectively were not statistically different.

Time course of synthase activation and Glc-6-P formation in hepatocytes from fasted rats

Fig. 3 (right panel) shows that the Glc-6-P concentration in freshly isolated hepatocytes (zero time) from fasted obese rats is twice that in the lean group. This observation is in agreement with the Glc-6-P levels that we measured in fasted-rat livers *in vivo* (van de Werve & Jeanrenaud, 1987). When the cells were incubated in the presence of 50 mM-glucose, there was no significant change with time in the Glc-6-P content of lean-rat hepatocytes, but there was a transient increase in cells from obese rats. These changes are compared with synthase activation in the same conditions (Fig. 3, left panel: data from Fig. 2).

At all glucose concentrations investigated, in fasted- or fed-rat hepatocytes, the Glc-6-P concentrations in the obese group were always at least twice those in the lean group (results not shown).

Interconversion of phosphorylase and glycogen synthase in Sephadex G-25 filtrates

In order to investigate the effect of phosphatases, without interference by kinases, on phosphorylase and synthase interconversion, liver extracts were filtered on Sephadex G-25. This procedure removes small molecules, including ATP, thus preventing kinases from being active in this preparation. Fig. 4 shows that there was a sequential inactivation of phosphorylase and activation of synthase upon incubation of liver filtrates from fed animals. The activation of synthase occurred after a lag

Fig. 3. Effect of incubation of fasted-rat hepatocytes with 50 mM-glucose on the levels of synthase *a* and of Glc-6-P

Hepatocytes from fasted lean (○) and obese (●) rats were incubated with 50 mM-glucose. Synthase *a* and Glc-6-P content were measured in samples collected and processed as described in the Experimental section. Values are means $\pm$ S.E.M. for three cell preparations in each group. All Glc-6-P values (lean versus obese) and those between 10 and 40 min versus 0 min in the obese group were statistically different from each other ($P < 0.05$).

Fig. 4. Interconversion of glycogen synthase and phosphorylase in liver filtrates

Liver extracts from lean (○, △) and obese (●, ▲) fed or fasted rats were filtered on Sephadex G-25 and incubated at 20 °C as described in the Experimental section. ○, ●, Synthase *a*; △, ▲, phosphorylase *a*. Values are means ± S.E.M. for four liver preparations per animal group and nutritional condition.

Fig. 5. Correlation between the activity of phosphorylase *a* and synthase *a* in liver preparations

Values of phosphorylase *a* from liver preparations as indicated are plotted versus the corresponding values of synthase *a*. (a) Isolated hepatocytes from experiments in Fig. 1; (b) isolated hepatocytes from experiments in Fig. 2; (c, d) liver filtrates from experiments in Fig. 4; ○, lean rats; ●, obese rats.

period of about 35 min, the time required for the almost complete inactivation of phosphorylase. In filtrates prepared from fasted-rat livers, synthase activation proceeded immediately, without lag period. Similar results were obtained when imidazole buffer was replaced by glycylglycine buffer. Synthase activation was similar in lean- and obese-rat liver filtrates in each nutritional group, indicating that in the absence of active kinases and of low-molecular-mass modulators the rate of endogenous synthase phosphatase was comparable.

Relationship between phosphorylase *a* and synthase *a* in hepatocytes and liver filtrates

Fig. 5 shows the inverse correlation between the activities of phosphorylase *a* and synthase *a* that was observed previously *in vivo* (Stalmans *et al.*, 1974), in isolated hepatocytes and in liver filtrates (Hue *et al.*, 1975; van de Werve *et al.*, 1983). In liver preparations from fed lean rats (Figs. 5a and 5c), there is an inverse relationship between the two activities. This feature is

Fig. 6. Correlation between the concentration of UDP-Glc and synthase *a* in hepatocytes

Two hepatocyte preparations from fasted lean (○) and obese (●) rats were incubated for 30 min in the presence of various concentrations of glucose (0–60 mM). Synthase *a* and UDP-Glc were measured as described in the Experimental section.

explained by the inhibition of synthase phosphatase by phosphorylase *a*. The threshold value of phosphorylase *a* below which synthase activation proceeds is close to 3 units/g of liver (see also Fig. 1). In contrast, in preparations from fasted lean animals, the extent of phosphorylase inactivation is smaller (Figs. 2 and 5b), and the threshold is much higher than 3 units/g of liver and less marked (Figs. 5b and 5d).

The salient observation here, however, is that the threshold value of phosphorylase *a* is markedly increased in hepatocytes from obese (fed or fasted) rats, but not in liver filtrates from the same animals (Fig. 5).

Correlation between the concentration of UDP-Glc and synthase *a* in fasted-rat hepatocytes incubated with glucose

Fig. 6 shows a linear negative correlation between the amount of synthase *a* and the UDP-Glc content in fasted lean- and obese-rat hepatocytes incubated with various concentrations of glucose. The elevated synthase *a* values in obese-rat hepatocytes were always matched with higher UDP-Glc concentrations than in the lean group. However, the slopes of the regression lines were similar in the lean and obese groups, indicating that, for a given increment in glycogen synthase *a*, the same amount of UDP-Glc was utilized.

DISCUSSION

Activation of synthase in fed- and fasted-rat hepatocytes

The time course of activation of synthase was quite different in fasted compared with fed lean rat hepatocytes (Figs. 1 and 2). The transient nature and almost complete reversal of synthase activation in fasted-rat cells indicates that the interconversion of synthase shifts from a situation where the activities of phosphatases prevail (activation) to one where that of kinases (inactivation) is predominant. Such a shift is not observed in hepatocytes from fed animals, where synthase activation leads to a steady state of the synthase *a* level. The cause(s) for the shift are unknown, but could be related to the presence of synthase kinase(s) which should then be more active in the fasted state, that is without glycogen. One obvious candidate would be protein kinase C, because this kinase is inhibited by glycogen (Ahmad *et al.*, 1984) (which would explain the lack of shift in the fed state). In fasted-obese-rat hepatocytes there is also a tendency for synthase activation to be reversed, but without resulting in an almost complete reversal as in the lean-rat cells (fig. 2). Therefore

part of the enhanced activation of synthase in fasted-obese-rat hepatocytes could be due to impaired synthase kinase(s) as suggested by other observations made in our laboratory (van de Werve & Massillon, 1990).

Our results are different from a previous report (Hue *et al.*, 1975) where synthase activation by glucose was larger in fasted than in fed-rat hepatocytes. The comparison is, however, difficult to make, because the experimental conditions here were not the same as those in the earlier study. Another strain of rats (Zucker versus Wistar), of a different sex (female versus male), was used, the time scale of hepatocyte incubation was longer (60 min versus 20 min), and the length of fasting was shorter (15 h, not 12–30 h).

Control of synthase activation by phosphorylase *a*

It appears that the sequential inactivation of phosphorylase and activation of synthase demonstrated previously (Stalmans *et al.*, 1971; Hue *et al.*, 1975; Witters & Avruch, 1978; van de Werve, 1981; van de Werve *et al.*, 1983; Bollen *et al.*, 1983; Mvumbi *et al.*, 1983, 1985; Alemany & Cohen, 1986) operates only in the fed liver. In the fasted glycogen-depleted liver, synthase phosphatase is no longer inhibited by phosphorylase *a* (Fig. 5) (Mvumbi & Stalmans, 1987; Schelling *et al.*, 1988).

Since the threshold value of phosphorylase *a* is higher in fed-obese-rat hepatocytes (Fig. 5a), it means that other yet-undefined factors besides glycogen depletion can weaken the inhibitory effect of phosphorylase *a* on synthase activation. The fact that the threshold is normal in liver filtrates from fed obese rats suggests that a labile or small compound (maybe Glc-6-P) might cause some de-inhibition of synthase phosphatase. Such a mechanism may also contribute to the enhanced activation of synthase.

Control of glycogen synthase phosphatase by Glc-6-P

Margolis (1987) reported increases in synthase- and phosphorylase-phosphatase activities in obese Zucker-rat liver *in vitro*. Our results do not indicate such enhanced synthase phosphatase in liver filtrates. Also, phosphorylase phosphatase activities do not seem to be different in filtrates and in cells from lean and obese animals (Fig. 4). One possible explanation for this discrepancy could be different experimental conditions of measurement of synthase phosphatase *in vitro*. The use of exogenous (skeletal muscle) substrates for the measurements of hepatic phosphatases *in vitro* (Margolis, 1987) differ from our assay with endogenous synthase. Newman & Curnow (1985) indeed reported that the cytosolic form of liver synthase phosphatase did not have the same properties towards muscle and liver synthase *b*. Stalmans & Bollen (1987) recommend the use of hepatic synthase *b* to measure synthase phosphatase in liver. Further characterization of the phosphatases and their substrates *in vitro* is required to evaluate the contribution of their intrinsic activity to the observed changes in hepatocytes.

In liver glycogen-particle preparations from rats pretreated with glucagon, Gilboe & Nuttall (1982) observed that synthase phosphatase was stimulated by Glc-6-P ($K_{0.5}$ 0.14 mM). This property seems to play a role in the intact hepatocyte as well. Ciudad *et al.* (1986, 1988a,b), using hepatocytes isolated from fasted rats, showed a positive correlation between the intracellular concentration of Glc-6-P and the activation of glycogen synthase. In fasted-rat hepatocytes (the present work), the extent of synthase activation was parallel to the Glc-6-P content (Fig. 4), suggesting that synthase phosphatase could be stimulated by the hexose phosphate.

Glc-6-P concentrations were higher in hepatocytes from fasted obese rats than in those from fasted lean rats, despite similar phosphorylase *a* levels in both groups of animals and at all

glucose concentrations investigated. This observation might be explained by the reported (van de Werve & Jeanrenaud, 1987; Table 1 in the present work) presence of glycogen in livers of fasted obese rats but not in those of fasted lean rats. It is also possible that metabolites (e.g. fructose 6-phosphate or glucose 1-phosphatase, as suggested by Newman & Curnow (1985) and Newman *et al.* (1987). Owing to the inhibitory action of Glc-6-P on synthase kinase(s) (De Wulf & Hers, 1968), a decreased synthase inactivation cannot be discounted. In this respect, it must be recalled that synthase inactivation by phorbol myristate acetate, a stimulator of protein kinase C, was impaired in hepatocytes from fasted genetically obese *fa/fa* rats (van de Werve *et al.*, 1987; van de Werve & Massillon, 1990).

In summary, our results show a marked increase in synthase activation in hepatocytes from fasted obese rats. This increase could result from a concerted effect of elevated Glc-6-P to stimulate synthase phosphatase and to inhibit synthase kinases. Our findings are also consistent with a possible impairment of synthase kinase(s), as we suggested previously (van de Werve *et al.*, 1987; van de Werve & Massillon, 1990).

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